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Cauda epididymal spermatozoa of the rufous hare wallaby, *Lagorchestes hirsutus* (Metatheria, Mammalia) imaged by electron and confocal microscopy

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Abstract

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The ultrastructure of mature *Lagorchestes hirsutus* spermatozoa is described for the first time, revealing unusual aspects of sperm structure in macropodid species. The sperm head is ovoid rather than cuneiform, lacks a ventral nuclear groove and has an acrosomal distribution over approximately 85–90% of its dorsal surface. Immediately adjacent to the nuclear membrane the peripheral nucleoplasm in most spermatozoa form an irregular series of distinctive evaginations previously not described in the spermatozoa of any other marsupial. The midpiece is extremely thickened and short, containing no helical network or peripheral plasma membrane specializations. Axonemal structure is unspecialized with no connecting lamellae; dense outer fibres are closely adherent to axonemal doublets. The sperm morphology of this species is highly aberrant in comparison to other macropod taxa and supports the retention of *Lagorchestes* as a distinctive genus. In light of this new information, skeletal and serological data should be re-evaluated to determine the true taxonomic and phylogenetic position of this species.

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Introduction

The rufous hare wallaby is a member of the metatherian family Macropodidae. It is a small, solitary, nocturnal wallaby, weighing up to 2 kg. In the wild it is now restricted to the Bernier and Dorre Islands off the coast of Australia but on the mainland experimental reintroduction programmes exist. We here investigate the structure of its spermatozoon and implications of this for the phylogenetic relationships of this endangered mammal.

Early light microscopic studies of the sperm structure of 23 macropodid species revealed a significant degree of heterogeneity in this taxon (Cleland in Rodger 1978); the sperm dimensions of head length, midpiece length and principal-piece width being the most variable. Cleland considered sperm head morphology amongst the Macropodidae distinctive enough for the identification of individual species, but found no grouping of sperm morphologies that reflected the current understanding of macropodid taxonomy (Rodger 1978); the only exception being spermatozoa from two extant *Lagorchestes* species that were morphologically distinctive.

Since Cleland's early investigation there have been numerous studies that have investigated the ultrastructure of macropodid spermatozoa, particularly with respect to phylogenetic implications (e.g. Harding *et al.* 1979; Harding 1987; Temple-Smith, 1987; Jones 1989; Taggart *et al.* 1995), but to date, no such observations of *Lagorchestes* spermatozoal ultrastructure. This study reports the first electron and confocal microscopic observations of mature spermatozoa from the cauda epididymides of *Lagorchestes hirsutus*, documenting the highly distinctive features of this spermatozoon.

Materials and Methods

Animals

Cauda epididymal spermatozoa were recovered from a 3-year-old and an 11-year-old *Lagorchestes hirsutus* (Gould, 1844) housed at Western Plains Zoo, Dubbo, New South Wales. The younger male was euthanased after sustaining severe physical trauma but was otherwise in healthy condition. The older male was euthanased following a prolonged period of idiopathic ill-thrift. Both animals were originally part of a central Australian captive population located at the Alice Springs Desert Park, Northern Territory.

Microscopy and sperm preparation

Immediately prior to euthanasia and while under gaseous anaesthesia, the testicles of the older male were removed and the cauda epididymides was processed for standard electron microscopy as described by Johnston *et al.* (1995). Testicular and epididymal tissue of the younger male was only obtained for electron microscopy after the tissue had been prepared for cryopreservation at the Animal Gene Storage Resource Centre of Australia (Monash Institute of Reproduction and Development, Clayton, Australia). The cryopreservation protocol consisted of storing the reproductive tissue at 4 °C for 2 days prior to freezing in 10% glycerol in phosphate-buffered saline at 10 °C/min. However, tissue from this animal was adequate to confirm the major ultrastructural features observed in the other male.

For light microscopic observations, spermatozoa were teased from the cauda epididymis and their dimensions were determined using Nomarski interference/phase contrast microscopy (1000×). With the aid of a calibrated eye-piece micrometer, the head, midpiece and flagellar dimensions of 40 spermatozoa were measured.

Visualization of the extent of acrosomal coverage of spermatozoa in this species was achieved by means of confocal microscopy. In preparation for confocal microscopy, a concentrated suspension of cauda epididymal spermatozoa was dispensed on to gelatinized slides and left to settle overnight. Following three washes in 0.1 M phosphate buffer (pH 7.2), spermatozoa were stained overnight with 2 μg/mL of propidium iodide. Prior to mounting in glycerol jelly, excess propidium iodide was removed using a further three washes of phosphate buffer. The advantage of this particular staining technique was that it provided differential staining of DNA material, so that the intact acrosome on confocal observation appeared as a shadow on the fluorescing sperm head. Spermatozoa were examined with a BioRad MRC 600 Krypton Argon Laser Zeiss Axioskop under oil immersion using an excitation wavelength of 488 nm and a green filter that produced an emission spectrum of 522–535 nm.

Results

Sperm dimensions

Sperm head length, width and depth measurements were 6.0 ± 0.1 μm (n = 40), 3.3 ± 0.1 μm (n = 28) and 2.3 ± 0.1 μm (n = 12), respectively; sperm head to width ratio was 1.9. Midpiece length and diameter were 5.1 ± 0.1 μm (n = 30) and 2.0 ± 0.1 μm (n = 40), respectively; midpiece length as percentage of the total sperm length was 5.8%. Total sperm length was 88.4 ± 0.3 μm (n = 40).

Sperm head ultrastructure

The sperm head of *Lagorchestes hirsutus* was ovoid rather than cuneiform as in most macropodids, was dorso-ventrally flattened and lacked a ventral groove (Figs 1A and 2A,B). The head of the mature spermatozoon was typically aligned parallel or slightly oblique to that of its longitudinal axis. Approximately one-sixth of the posterior sperm head overlay the midpiece; this nuclear orientation was accommodated by a distinctive sculpturing of the midpiece cranially (Fig. 1A).

While the nucleoplasm of most cauda epididymal spermatozoa was evenly condensed, a distinctive ultrastructural feature of the head in some spermatozoa was the appearance of an unusually high number and extent of evaginations of nucleoplasm [Fig. 1A(a,b),B].

The implantation fossa of the sperm head was simple, being only a slight indentation on the medial ventral aspect of the sperm head, associated with a simple basal plate of electron-dense material (Fig. 1B).

Acrosomal ultrastructure

The acrosome of the cauda epididymal spermatozoon was condensed and covered approximately 85–90% of the dorsal surface of the nucleus. Electron micrographs through the sperm head [Fig. 1A(a,b),B], as well as confocal microscopic observations (Fig. 2C–E) revealed that the acrosome occurred centrally on the dorsal surface of the nucleus and tapered peripherally.

Sperm flagellum

The connecting piece of the spermatozoon appeared as an unspecialized elongated tapered structure with a rounded capitulum, forming a shallow 'ball and socket' articulation with the implantation fossa. Lateral views of the connecting piece indicated that this structure was usually asymmetrically situated at the terminal portion of the axoneme and was surrounded by a cylindrical sleeve of striated columns.

The midpiece was extremely short, thick (Figs 1A and 2A,B) and essentially circular in cross-section (Fig. 1c). In sagittal section the mitochondrial sheath was composed of six gyres of large flattened mitochondria containing large

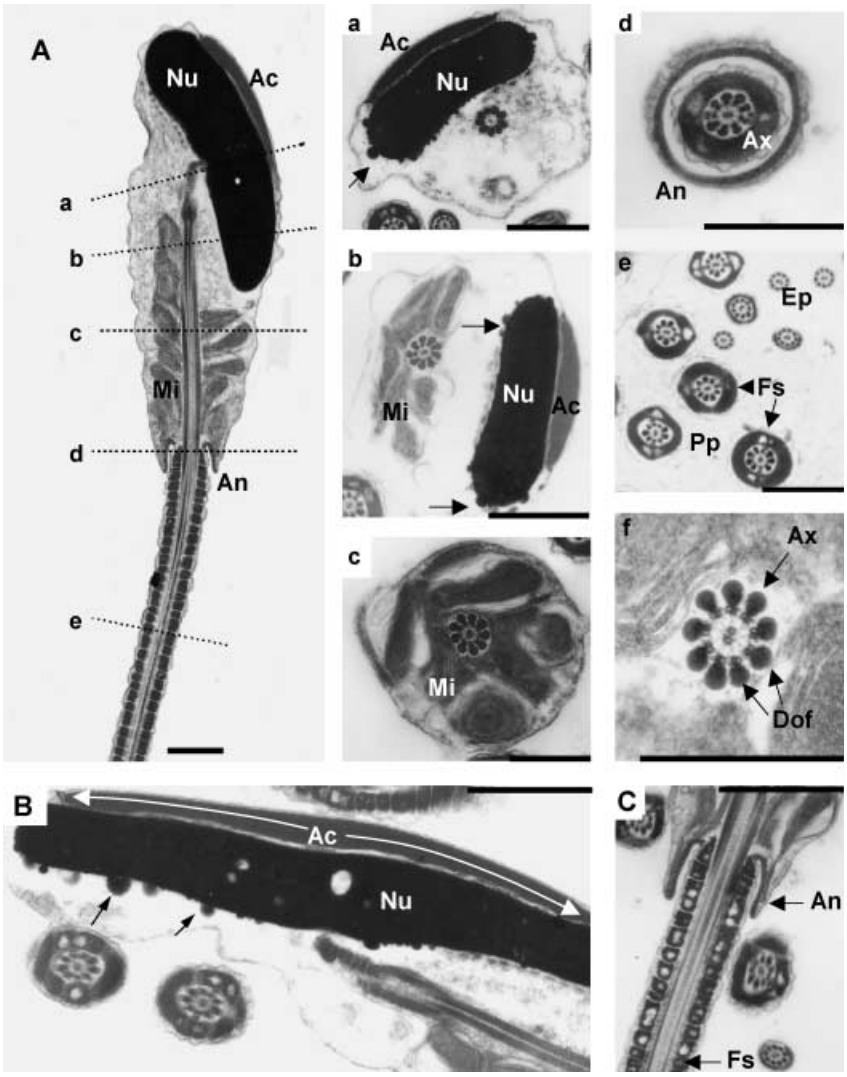

Fig. 1—**A**. Longitudinal section of a cauda epididymal rufous hare wallaby spermatozoan, (a)–(f) illustrate various cross-sections along the length of the mature spermatozoan. —**B**. Sagittal section of the rufous hare wallaby sperm head showing acrosomal coverage on the dorsal portion of the nucleus. —**C**. Longitudinal section of the annular region of the mature spermatozoa of the rufous hare wallaby. Ac, acrosome; An, annulus; Dof, dense outer fibres; Ep, endpiece; Fs, fibrous sheath; Mi, mitochondrial sheath; Nu, nucleus; Pp, principal piece; arrows, pointing to evaginations of nucleoplasm. Scale bar = 1 μm.

concentric cristae that appeared to show slightly degenerative changes in some micrographs; these changes were not considered to be fixation artefacts.

In the midpiece region of the spermatozoon, the axoneme was unspecialized, with no connecting lamellae, so that the dense outer fibres closely overlay the axonemal doublets (Fig. 1f). While there was neither fibre network nor plasma membrane specialization in the midpiece region, the caudal extremity of the mitochondrial sheath formed a thickened skirt in association with an elongated caudally reflexed annulus (Fig. 1C).

The cranial portion of the principal piece was effectively circular in cross-section and composed of a thickened fibrous sheath [Fig. 1A(e)]; dense outer fibres were closely associated with all nine axonemal microtubule doublets. However, in the more caudal regions of the principal piece, the underlying fibrous sheath became progressively flattened dorso-laterally to give rise to longitudinal columns with double fenestrations (not visible on all) and an associated loss of dense outer fibres. In the terminal portion of the sperm flagellum the fibrous sheath was absent and a distinct endpiece [Fig. 1A(e)] was formed which was approximately 7 μm in length.

Discussion

The sperm dimensions from *Lagorchestes hirsutus* described in this study were the smallest thus far described for any macropodid; in particular, the midpiece region was the shortest reported of any Australian marsupial (Rodger 1978; Taggart 1994; Temple-Smith 1994), ringtail possums being the only Australian marsupials with a midpiece of comparable proportions. Thus, light, transmission electron and confocal microscopy of these spermatozoa confirm Cleland's original observations (Rodger 1978), that they have different morphometrics to those of other macropodids. The functional significance of such sperm dimensions with respect to motility

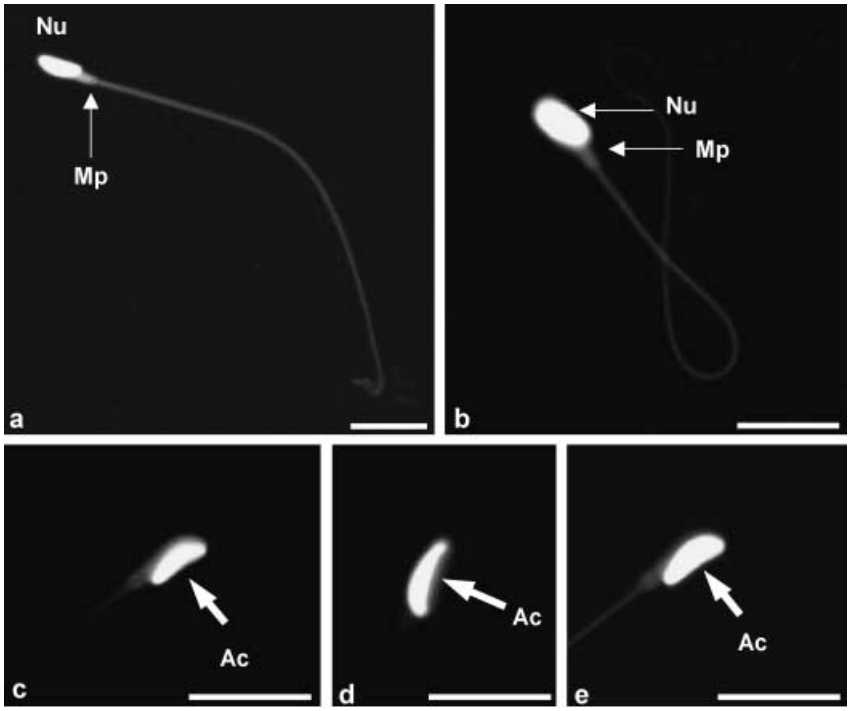

Fig. 2—Confocal micrographs of rufous hare wallaby spermatozoa illustrating: —**A**, **B**. the short and thickened midpiece and—**C**–**E**. orientation of the acrosome which covers approximately 90% of the dorsal nuclear surface. Ac, acrosome; Mp, midpiece; Nu, nucleus; arrows, pointing to the shadow left by the nonfluorescing acrosome. Scale bar = 10 μm.

patterns and fertilization biology remain to be investigated. Amongst the Diprotodontia, the length : diameter dimensions of the midpiece and the proportional cross-sectional area of the axoneme to midpiece in *L. hirsutus* closely resemble that described for the *Pseudocheirus* group (Harding *et al.* 1979).

The sperm head of *L. hirsutus* is ovoid and without a distinct ventral groove, characteristics that this species appears to share with no other macropodid spermatozoa so far described. In order for the sperm head to achieve a 'streamlined' alignment relative to the long axis of the spermatozoon, the anterior extremity of the midpiece has been heavily sculptured.

While the nucleoplasm of mature *L. hirsutus* spermatozoa generally appears condensed, the sperm head of some spermatozoa shows what appears to be evagination of the nucleoplasm periphery beneath or in association with degeneration of the nuclear membrane. This specific phenomenon has not been previously reported in marsupial spermatozoa and it seems unlikely to be a fixation artefact. The same phenomenon has also been observed below the intact nuclear membrane of well-fixed testicular spermatozoa (Johnston unpublished observations). While the spermatozoa of some dasyurid species show nuclear modification or protrusions of nucleoplasm around the periphery of the nucleus (Harding *et al.* 1979), there is no published information of nucleoplasm modification in the Macropodidae and the phenomenon in *L. hirsutus* appears distinctively 'exocytotic'. As Harding *et al.* (1979) have noted for the Dasyuridae, perhaps nucleoplasm peripheral evagination is an expression of delayed nuclear condensation, representing a method of disposal of excess nucleoplasm. Preliminary observations of both testicular and caput epididymal spermatozoa (Johnston, unpublished observations) indicate that the incidence of nucleoplasm extrusion is more extensive and may possibly be part of the sperm maturation process in this species.

The acrosome of *L. hirsutus* epididymal spermatozoa is radically different to that of all other known macropodid spermatozoa save *Hypsiprymnodon moschatus* (Musky Rat-Kangaroo; Lloyd *et al.* 2002). The acrosome of *L. hirsutus* covers approximately 85–90% of the dorsal surface of the nucleus and is tapered only at its cranial and caudal extremities. The acrosome of *H. moschatus* also covers the dorsal nuclear surface but has a distinctive 'button-like' section extending to the rostral tip of the nucleus and only a thin plate-like portion extending caudally. By contrast the majority of kangaroo spermatozoa contain acrosomes that cover only 30–45% of the dorsal nuclear surface (Harding *et al.* 1987). In this respect the acrosome morphology of both species resembles more that found in the Dasyuridae, Peramelidae and Thylacomydiae (Temple-Smith 1994; Johnston *et al.* 1995).

The sperm mitochondrial architecture of *L. hirsutus* cauda epididymal spermatozoa consists of large flattened concentric lamellae often with an electron-dense centre. This particular mitochondrial morphology is not typical of any other macropodid spermatozoa so far described.

The axoneme in the midpiece region of the *Lagorchestes* spermatozoon is unspecialized with no connecting lamellae, so that the dense outer fibres closely overlie the axonemal doublets. In this respect the spermatozoa are unlike those of other macropodids which all have varying degrees of distinct separation between the dense outer fibres and the axonemal doublets (Harding *et al*. 1987). *Lagorchestes hirsutus* spermatozoa are also unlike other macropod spermatozoa in not possessing any form of fibre network in the midpiece region. They also possess an elongated strongly reflexed annulus similar to that described for *Pseudocheirus herbertensis* (Harding *et al*. 1987).

This study demonstrates that the spermatozoa of *L. hirsutus* have morphological characteristics (nuclear evaginations; large acrosomal area; absence of outer dense fibre lamellae; absence of fibre network in the midpiece; concentric cristae and dense core; strongly reflexed annulus) that differ from those of other Macropodidae. Sperm morphology appears to be consistent with *L. hirsutus* being an early macropodid branch that has evolved with a high degree of parallelism. In light of these new findings, the current skeletal (Flannery 1989) and serological (Kirsch *et al*. 1977; Burk and Springer 2000) data may need to be re-evaluated and supporting molecular data obtained to determine the true taxonomic and phylogenetic position of this species. Further studies of sperm morphology of extant congeneric *Lagorchestes* species (*L. conspicillatus* and *L. fasciatus*) are required to determine whether similar morphology is conserved throughout the genus.

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